

## Inhibition of Pneumococcal Carriage in Mice by Subcutaneous Immunization with Peptides from the Common Surface Protein Pneumococcal Surface Adhesin A

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Pneumococcal surface adhesin A (PsaA), a common protein expressed on all 90 pneumococcal serotypes, is a vaccine candidate. Three anti-PsaA monoclonal antibody phage display-expressed nonpeptides (15 mers), in various formulations as lipidated or nonlipidated multiantigenic peptides or as bi- or tripeptide constructs, were studied in a mouse nasopharyngeal carriage model to determine the inhibitory effect of induced antibodies on carriage of pneumococcal serotypes 2, 4, and 6B. Antibodies to each of the various peptides tested reduced carriage of the 3 serotypes. Reduction in carriage by nonlipidated multiantigenic peptide antibodies was highly variable (39%–94%; mean, 59%; standard deviation [SD], 20.2%); however, more-consistent results were observed in mice immunized with lipidated (56%–98%; mean, 69%; SD, 13.6%) and combination or bipeptide (55%–91%; mean, 76%; SD, 13.1%) formulations. These peptides are immunogenic, and their induced antibodies reduce carriage in mice. PsaA peptides demonstrate potential for being important new vaccines against pneumococcal carriage, otitis media, and invasive pneumococcal disease.

*Streptococcus pneumoniae* is a leading cause of morbidity and mortality and a major cause of bacterial pneumonia, meningitis, bacteremia, and otitis media in developed as well as developing countries [1–8]. The currently licensed vaccines available for immunoprophylactic control of these pneumococcal diseases are a first-generation 23-valent pneumococcal capsular polysaccharide (PS) vaccine and a second-generation 7-valent PS conjugate vaccine in which the PS is conjugated to the diphtheria protein CRM-197 [9]. In addition, several other investigational second-generation PS-protein conjugate vaccines, containing up to 11 serotypes, are presently in phase II and III trials in various parts of the world (The Gambia, Israel, Philippines, and South Africa) [10–14]. These pneumococcal vaccine formulations are based on the observation that antibodies to the PS protect against disease by enhancing phagocytosis [15].

The 23-valent PS vaccine covers ~90% of invasive pneumococcal disease in the United States, whereas the 7-valent conju-

gate vaccine covers ~80% of strains causing disease in children <5 years of age but only 55% of invasive pneumococcal disease among adults. However, the existence of ≥90 pneumococcal serotypes has complicated further development of these classes of vaccines. Although these vaccines cover the most prevalent serotypes found in the population at risk, they protect mainly against illness caused by those serotypes included in the vaccine. A limited amount of cross-protection among the serotypes has been observed with both the PS and the conjugate vaccine.

Alternate formulations of pneumococcal vaccines are under development. Considerable research is being conducted on potential third-generation common protein vaccines. Candidates under consideration are various pneumococcal cell-wall components and protein antigens, including pneumococcal surface adhesin A (PsaA), pneumococcal surface protein A, and the protein PdB, a recombinant nontoxic pneumolysin [16–19]. These pneumococcal proteins, which are T cell dependent, are common to all pneumococcal serotypes and are likely to be highly immunogenic in humans and to elicit immunologic memory. In contrast to current pneumococcal vaccines, third-generation vaccine candidates will provide protection to a broader target population, from infants to elderly individuals, and to those at high risk. Furthermore, it is hoped that these will not be geographically specific and will provide protection against all pneumococcal serotypes.

PsaA, a 37-kDa lipoprotein expressed by all 90 *S. pneumoniae* serotypes, has been extensively characterized [16, 17, 19–23]. It has been shown to be immunogenic and protective against invasive pneumococcal disease and intranasal carriage in mice [17, 20–23]. Mutation analysis studies have demonstrated that changes in PsaA protein affect the binding of *S. pneumoniae*, indicating that PsaA plays a critical role in bacterial adherence and virulence [24]. In addition, research has indicated that PsaA

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determinants and epitopes are either completely or partially exposed on the surface of the cell [16]. These characteristics make PsaA a highly desirable candidate for use in the development of a third-generation pneumococcal vaccine.

However, use of proteins for vaccine development has limitations. Proteins are macromolecules composed of numerous amino acids that form 3-dimensional complexes. Because of the coiling and twisting of these complexes, potential immunogenic epitopes may be sterically hidden, obscured, or blocked and thus prevented from eliciting an immunologic response. In addition, proteins may have components that give rise to unwanted side effects or produce immunogens that hinder the wanted immunoreactive effect.

One way to avoid the disadvantages of using proteins for vaccines is to identify, isolate, and synthesize immunoreactive peptides (epitopes) of interest from the protein. Such epitopes are short, small amino acid units of the protein that can be linear and that are easier and less costly to synthesize for mass production. The use of synthetic peptides makes it possible to elicit an immunologic response to epitopes that remain cryptic during natural infection. In addition, it makes available antigenic and/or immunogenic peptides that may be difficult to prepare in quantity from native sources. Furthermore, the use of peptides rather than large, cumbersome, and costly proteins provides the opportunity to formulate vaccines with combinations of more immunoreactive units. Immunoreactive peptides, therefore, have the potential for being used to produce vaccines of the fourth generation.

Recently, we identified, isolated, sequenced, and described, with use of anti-PsaA mouse monoclonal antibodies, 3 idiotypes (mimotopes) from a phage display library reported elsewhere [25]. PsaA peptides corresponding to these idiotypic sequences were shown to be 15 aa long and immunogenic in mice and demonstrated the ability to elicit an immunologic response that inhibited colonization of a pneumococcal serotype 2 isolate in a mouse nasopharyngeal (NP) carriage model [25]. Subsequently, we designed a study to determine whether peptides formulated from the sequence of these idiotypes would be viable candidates for the development of fourth-generation pneumococcal vaccines. This study, described herein, examined the inhibitory effects of antibodies induced by various peptide constructs formulated from these PsaA peptides on pneumococcal NP carriage in mice (i.e., ability of PsaA peptides to reduce the risk of acquiring pneumococci for carriage).

## Materials and Methods

**Bacterial isolates and growth conditions.** Three isolates of *S. pneumoniae*, representing serotypes 2, 4, and 6B, were used for NP carriage experiments. Serotype 2 (isolate PLN-D39), a pneumolysin-negative mutant of D-39, was provided by James Paton (Women's and Children's Hospital, North Adelaide, Australia) [26]. Serotypes

4 (isolate DS2341-94) and 6B (isolate DS1756-94), which are typical strains, were provided by Richard Facklam (Centers for Disease Control and Prevention, Atlanta). All 3 isolates have been used frequently in animal model studies, in our laboratory and elsewhere. To ensure that multiple experiments could be initiated from the same lot of cells, a standardized stock culture of each pneumococcal isolate was prepared, as described elsewhere [27]. On initiation of an experiment, stock cells were cultured on blood agar (trypticase soy agar supplemented with 5% defibrinated sheep blood; BBL Microbiology Systems), transferred to brain-heart infusion broth (BBL Microbiology Systems) supplemented with 10% Levinthal's basal medium (BBL Microbiology Systems), and manipulated for animal inoculation, as described elsewhere [27].

**Animals.** Adult Swiss-Webster mice (ND-4; female; 8 weeks old) were obtained from Harlan Sprague Dawley. On arrival, mice were arranged in groups of 8 per cage. All animals were housed under standard conditions (25°C, relative humidity ~40%) with food and water available ad libitum. All mice were allowed to acclimate to their new environment for 1 week before experimentation.

**Peptide synthesis.** Three base peptides, designated "P1," "P2," and "P3," were used as templates to construct multiantigenic peptides (MAPs) and polypeptides (figures 1–4). The amino acid sequences of these peptides were defined from the 3 PsaA idiotypes mentioned above and have been described elsewhere (figure 1) [25]. In brief, the sequences for these base peptides were 15 aa in length. The motifs for these were found to align themselves along the PsaA protein strand at different locations and to have varying degrees of homology to the native protein. The P1 motif was aligned in region aa 132–146 and had a homology of 2 aa at positions aa 140 and 141. The alignments for the P2 and P3 motifs were found to be farther along on the protein strand at regions aa 206–220 and aa 250–275, respectively. The motif for P2 was found to have a 3-aa homology with the aligned region at positions aa 214, 215, and 216. Unlike the P1 and P2 motifs, the homology of the P3 motif consisted of 6 aa and was not continuous. Within the aligned region, the homologies occurred in 4 separate areas and were positioned at aa 253, 257, 260–261, and 266–267.

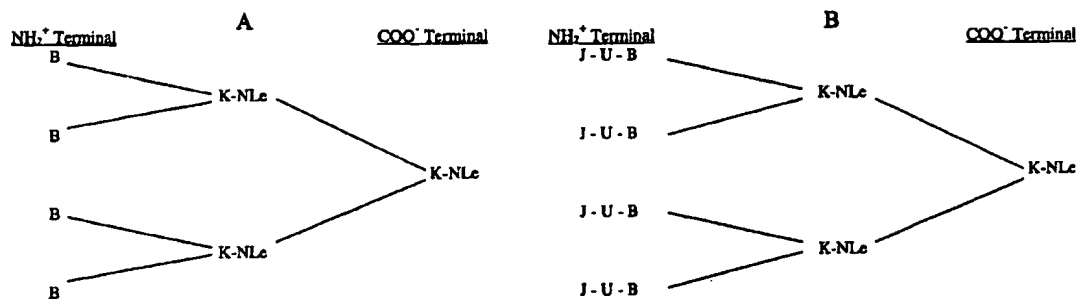
By using the sequences of the 3 base peptides as templates, three 4-arm, homologous MAPs in both the lipidated and nonlipidated forms were synthesized, along with nonlipidated bipeptide (4-arm, heterologous) and tripeptide (3-arm, heterologous) constructs (figures 2–4) and were studied in a mouse NP carriage model. The MAPs were synthesized on an ACT 396 multiple peptide synthesizer (Advanced ChemTech) by use of standard and modified Fmoc protocols, as described elsewhere [28–30]. The bi- and tripeptide

P1: T-V-S-R-V-P-W-T-A-W-A-F-H-G-Y

P2: R-S-Y-Q-H-D-L-R-A-Y-G-F-W-R-L

P3: L-V-R-R-F-V-H-R-R-P-H-V-E-S-Q

**Figure 1.** Amino acid sequences of 15-mer base peptides (P1, P2, and P3, described in Materials and Methods) used as templates to construct multiantigenic peptides and polypeptides [25]. A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; L, leucine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.



**Figure 2.** Basic structure of 6 multiantigenic peptides, 3 nonlipidated (A) and 3 lipidated (B). B represents three 15-aa base peptides (P1, P2, and P3, described in Materials and Methods) (figure 1) [25]. "J" represents the palmitoyl (palmitic acid) group, which is linked to the N-terminal of base peptides through a 3-aa chain linker (cysteine-serine-serine), designated "U" [31]. For P43 and P46, B = P1; for P44 and P47, B = P2; and for P45 and P48, B = P3. K-NLe, lysine-norleucine.

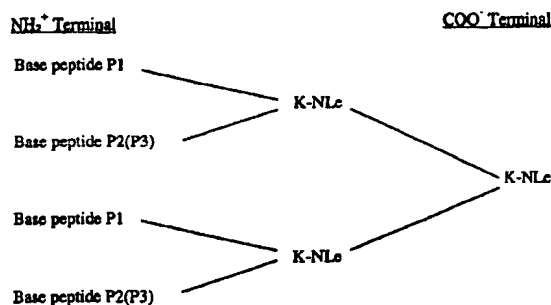
constructs were synthesized by the differential deprotection of Fmoc-Lys(Dde) or Fmoc-Lys(ivDde) (Novabiochem) with piperidine and hydrazine, using the method of Tam [31]. The crude peptides were analyzed for fidelity of synthesis by matrix-assisted laser desorption-ionization time-of-flight mass spectroscopy, followed by 1 or more of the following procedures: amino acid analysis, high-performance liquid chromatography, capillary electrophoresis, or peptide sequencing. When necessary, the peptides were purified by semiprep high-performance liquid chromatography, using acetonitrile-water-trifluoroacetic acid buffer systems.

Structurally, the MAPs contain 4 branches that are homologous to each other and consist of P1, P2, or P3. Lipidated forms (designated "P43," "P44," and "P45") and nonlipidated forms (designated "P46," "P47," and "P48") are identical in structure, except that the former have a monosubstituted palmitoyl residue attached at the N-terminal of each base peptide unit by the 3-aa linker cysteine-serine-serine (figure 2). The MAPs were lipidated with palmitic acid to study the effect of an adjuvant on peptide immunogenicity and subsequent influence on NP carriage. P43 and P46 were previously referred to as peptides "43" and "46" [25]. Bipeptides, referred to as "P79" and "P80," are a combination of the base peptides (P1 and P2 or P1 and P3; figure 3). P79 contains 2 units each of P1 and P2, whereas for P80, P2 is replaced with P3. The tripeptide, identified as "P81" and consisting of 3 branches, is made up of 1 unit each of the base peptides (figure 4). In all cases, the peptide branches were synthesized on either a 3-arm or a 4-arm lysine-norleucine core (figures 2-4).

**Mouse immunization and challenge.** A mouse NP carriage model, described earlier, was used to determine the effect of immunogenic PsaA peptides on inhibition of NP pneumococcal carriage [32]. Mice were immunized according to a 3-dose regimen. Test mice ( $n = 8$  for each peptide or peptide combination) received an initial dose of 100  $\mu$ g at day 0, followed by booster doses of 50  $\mu$ g of the appropriate peptide at 3 and 5 weeks after the initial dose. The lipidated MAPs were suspended in 100  $\mu$ L of sterile 0.01 M PBS (pH 7.2); all other peptides were mixed with 100  $\mu$ L of the adjuvant alum (alhydrogel-2%; catalog A1090B; Accurate Chemical and Scientific) at 6.3 mg/mL in PBS, to enhance the immunogenicity of the peptide. Control mice ( $n = 8$ ) were similarly

immunized but without peptide. Each mouse was immunized subcutaneously between the shoulders. One week after the final booster immunization, all mice were challenged intranasally with  $1 \times 10^6$  to  $3 \times 10^6$  cfu of the appropriate *S. pneumoniae* isolate suspended in 10  $\mu$ L of 0.9% sodium chloride buffer (Abbott). Challenge inoculum was diluted serially 10-fold and was plated on blood agar plates to determine challenge dose.

**Nasal wash and culturing.** Five days after challenge, each mouse was euthanized, and the NP cavity was washed with sodium chloride buffer, as described by Wu et al. [33]. In brief, 100  $\mu$ L of wash was collected in a tube containing an equal volume of sodium chloride buffer (1:2 dilution) and then was diluted serially 3-fold to a final dilution of 1:486. Fifty microliters of each dilution was cultured on selective media (blood agar plates supplemented with 2.5 mg/L gentamicin to suppress growth of organisms other than *S. pneumoniae*). Cultures were incubated overnight at 37°C in an atmosphere of 5% CO<sub>2</sub> and high humidity. When possible, colonies were counted for each dilution, and the count was recorded. Carriage for a particular mouse was defined as the number of colony-forming units of challenge pneumococci in 50  $\mu$ L of collected nasal wash after counts for each dilution were adjusted to a nondi-



**Figure 3.** Basic structure of bipeptides P79 and P80. Base peptides (P1, P2, and P3, described in Materials and Methods) are linked together by the 2-aa linker lysine-norleucine (K-NLe) to form a 4-branched bipeptide (figure 1) [25, 31]. P79 consists of 2 units each of base peptides P1 and P2. In bipeptide P80, P2 units are replaced with P3 units.

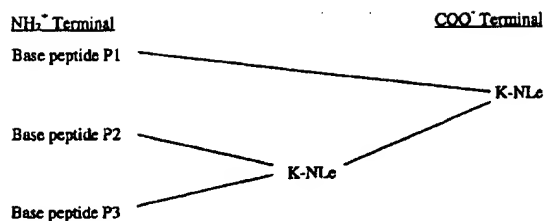


Figure 4. Basic structure of tripeptide P81. Base peptides (P1, P2, and P3, described in Materials and Methods) are linked together by the 2-aa linker lysine-norleucine (K-NLe) to form a 3-branched tripeptide (figure 1) [25, 31].

luted level and averaged. Sensitivity of carriage assay was 40 cfu/mL of nasal wash.

**Statistical methods.** Inhibition of pneumococcal carriage was determined by comparing the numbers of pneumococcal bacteria colonizing the NP area between immunized test mice and their paired immunogen-free control mice. Differences between groups were determined by the Mann-Whitney rank sum test, with level of significance set at  $P < .05$ . Statistical calculations were done with SigmaStat software (version 2.0; Jandel Scientific). Analysis was based on data generated from a single trial for each pneumococcal serotype and immunogen formulation.

## Results

**Effect of MAP immunization on inhibition of NP carriage.** In preliminary experiments, an immunologic response to MAP P43 was observed in mice (titers measured against naïve PsA consistently  $\geq 1:51,200$ ) when the animals were immunized according to the schedule described above (data not shown). On the basis of these results, pneumococcal carriage inhibition experiments were initiated on adult mice with the peptides described. When mice were immunized with nonlipidated MAP P46, P47, or P48 and challenged with *S. pneumoniae* serotype 2, 4, or 6B, reduction in NP carriage was observed for all and varied widely, from 39% to 94% (mean, 59%; SD, 20.2%), compared with the control group and as defined by the mean number of colony-forming units of challenge pneumococci colonizing the NP cavity (table 1). For those mice immunized with P46, reductions in NP colonization of 69% ( $P = .04$ ) and 94% ( $P < .01$ ) were observed for serotypes 2 and 4, respectively. For serotype 6B, the reduction was lower, 45%, but not significant ( $P = .11$ ). Similarly, for P47-immunized mice, reduction in NP carriage ranged from 40% to 88%, with serotype 4 being inhibited the most. In addition, when mice were immunized with P48, the numbers of pneumococci colonizing the NP cavity were reduced by  $\geq 39\%$  for all 3 serotypes. Similar but less variable results were seen when mice were immunized with lipidated MAP P43, P44, or P45 (table 2). Reductions in NP carriage were observed for all 3 serotypes and were  $\geq 56\%$  (mean, 69%; SD, 13.6%). The largest decreases in carriage were again observed in mice challenged with serotype 4. Antibodies induced by these 3 lipidated MAPs in serotype 4-challenged

mice significantly ( $P < .05$ ) reduced carriage by as much as 98% and no less than 74%. Lipidated MAP-immunized mice that were challenged with either serotype 2 or 6B experienced a reduction in pneumococcal carriage of 56%–65%.

**Effect of immunization with MAP combinations or polypeptides on inhibition of NP carriage.** The encouraging effects on inhibition of NP carriage produced by the immunologic response to the MAPs prompted further study of MAP combinations and polypeptide constructs. In all experiments in which mice were immunized with MAP combinations (P43 and P44 or P43, P44, and P45) or bipeptides (P79 or P80), inhibitions of NP carriage of  $\geq 55\%$  (mean, 76%; SD, 13.1%;  $P < .05$ ) were observed (table 3). Bipeptide-immunized mice challenged with any of the 3 serotypes experienced a reduction in NP carriage of  $\geq 68\%$ . Similar observations were seen in mice immunized with the tripeptide construct P81. These mice generated an immunologic response that reduced NP carriage of serotypes 2 and 4 by 53% and 82%, respectively ( $P < .05$ ). Likewise, similarly immunized mice experienced a reduction in carriage of 49% ( $P = .14$ ) when challenged with serotype 6B. Overall, an average decrease in pneumococcal carriage of 75% was observed in mice immunized with polypeptide constructs and challenged with serotype 2, 4, or 6B.

## Discussion

The currently licensed pneumococcal vaccines, a 23-valent PS and a 7-valent PS-protein conjugate vaccine, along with several other polyvalent PS-protein conjugate vaccines currently undergoing trials, have serious limitations. The 23-valent vaccine is not licensed for children  $< 2$  years of age because of poor immune response. It induces protection in persons  $> 65$

Table 1. Inhibition of nasopharyngeal carriage of *Streptococcus pneumoniae* by nonlipidated pneumococcal surface adhesin A multiantigenic peptides in a mouse model.

<i>S. pneumoniae</i> serotype, peptide	Mean cfu (% reduction)	P
2		
P46	284 (69)	.04*
P47	1545 (40)	.24
P48	1344 (39)	.20
4		
P46	47 (94)	$< .01^*$
P47	46 (88)	$< .01^*$
P48	1438 (42)	.49
6B		
P46	1114 (45)	.11
P47	1312 (63)	.13
P48	1134 (58)	.03*

NOTE. Data are mean no. of colony-forming units (cfu) per mouse ( $n = 8$ ) recovered in 100  $\mu$ L of wash from nasopharyngeal cavity. Mice were challenged intranasally with  $10^6$  cfu of *S. pneumoniae*.

\* Significant difference vs. control mice injected with either PBS or alum ( $P < .05$ ; Mann-Whitney rank sum test).

**Table 2.** Inhibition of nasopharyngeal carriage of *Streptococcus pneumoniae* by lipidated pneumococcal surface adhesin A multiantigenic peptides in a mouse model.

<i>S. pneumoniae</i> serotype, peptide	Mean cfu (% reduction)	P
2		
P43	2516 (56)	.13
P44	815 (64)	.11
P45	1766 (65)	.20
4		
P43	182 (74)	.048*
P44	23 (98)	<.01*
P45	142 (82)	<.01*
6B		
P43	1729 (63)	.02*
P44	1627 (58)	.01*
P45	544 (60)	.20

NOTE. Data are mean no. of colony-forming units (cfu) per mouse ( $n = 8$ ) recovered in 100  $\mu$ L of wash from nasopharyngeal cavity. Mice were challenged intranasally with  $10^6$  cfu of *S. pneumoniae*.

\* Significant difference vs. control mice injected with either PBS or alum ( $P < .05$ ; Mann-Whitney rank sum test).

years of age that wanes significantly after 5 years [34–37]. Vaccine efficacy for the 23-valent vaccine is limited by the T cell-independent nature of the PS antigen and the number of serotypes represented in the vaccine [35]. Also, because antibodies to PS have limited cross-reaction with other serotypes, protection from this vaccine and from the various polyvalent PS-protein conjugate vaccines licensed or undergoing trials is afforded only to vaccine serotypes and selected other serotypes. Furthermore, the conjugate vaccine preparations have not proved to be more immunogenic than the conventional 23-valent vaccine in limited evaluations made to date among adults >50 years of age. Finally, the primary antibody response induced by PS-protein conjugate vaccines continues to manifest T cell-independent characteristics, such as dominance of IgG2 over IgG1 response in human adults [38–40]. On the other hand, PsaA is a component of the *S. pneumoniae* cell wall, which is highly conserved among the different serotypes of this organism and plays a key role in bacterial adherence and virulence. Antibodies against PsaA have been shown to be cross-reactive among all serotypes of *S. pneumoniae* [16]. As a protein antigen, it is capable of eliciting a T cell-dependent immune response. These properties of PsaA make it an ideal candidate for vaccine development.

Epitope mapping is a convenient procedure with which to delineate antigenic determinants of a molecule. In a previous study, we isolated, sequenced, and described 3 peptides from PsaA [25]. In the current study, we used various formulations of these peptides (MAPs, alone in the lipidated or nonlipidated forms, in combination with each other, or as bi- or tripeptide constructs) to determine the inhibitory effect of the immunostimulated antibodies on pneumococcal NP carriage in mice.

Our results indicated that inhibition of NP carriage among the pneumococcal isolates tested varied widely when mice were

immunized with nonlipidated PsaA MAPs. However, this variability decreased and was accompanied by higher rates of reduction in NP carriage when mice were immunized with lipidated MAPs, MAP combinations, or polypeptide constructs.

The ability of induced antibodies to our synthesized PsaA MAPs and polypeptide constructs to inhibit NP carriage of pneumococci can be based on several factors. These factors involve the peptide amino acid sequence, the peptide dosage used during immunization, and the adjuvant used to enhance peptide immunogenicity. The peptides used as templates in this study for synthesizing the MAPs were described earlier and were characterized as having a 2–6-aa homology to the native PsaA protein [25]. In addition, these homologous amino acids did not necessarily form a continuous motif. Sequences chosen from a phage display library are selected by monoclonal antibodies on the basis of their affinity to the given monoclonal antibody [41, 42]. Although these homologous amino acids are essential for binding to monoclonal antibody, it is possible that the intervening amino acids in the native protein could play a critical role in its immunogenicity. Also, amino acids distant in the primary sequence but adjacent in the tertiary structure of the protein may possibly play a critical role in modulating the immune response. Thus, use of a peptide sequence with greater homology to the native protein might be promising and might improve the immunogenicity.

In the present study, effects of dose ranging and scheduling on immunogenicity and NP carriage inhibition were not examined. However, studies of the immunogenicity of synthetic peptides indicate that immunogenicity is a function of peptide con-

**Table 3.** Inhibition of nasopharyngeal carriage of *Streptococcus pneumoniae* by pneumococcal surface adhesin A multiantigenic peptide combinations, bipeptides, or tripeptides in a mouse model.

<i>S. pneumoniae</i> serotype, peptide(s)	Mean cfu (% reduction)	P
2		
P43 + P44	603 (55)	.03*
P43 + P44 + P45	606 (60)	.04*
P79	1247 (78)	.03*
P80	718 (87)	<.01*
P81	580 (53)	.03*
4		
P79	212 (83)	.01*
P80	466 (82)	<.01*
P81	45 (82)	<.01*
6B		
P79	301 (91)	<.01*
P80	476 (68)	.02*
P81	872 (49)	.14

NOTE. Data are mean no. of colony-forming units (cfu) per mouse ( $n = 8$ ) recovered in 100  $\mu$ L of wash from nasopharyngeal cavity. Mice were challenged intranasally with  $10^6$  cfu of *S. pneumoniae*.

\* Significant difference vs. control mice injected with either PBS or alum ( $P < .05$ ; Mann-Whitney rank sum test).

centration. A study of the peptide mimic of capsular polysaccharide of *Neisseria meningitidis* serogroup A (NmAPS) showed that the peptide is more immunogenic at lower concentrations. Optimal antibody response to NmAPS was observed in mice immunized with 1 and 5  $\mu$ g of peptide, and the serum samples from the group immunized with 5  $\mu$ g of peptide had the highest serum bactericidal assay titer [43]. In contrast, in a peptide mimic of capsular polysaccharide of *N. meningitidis* serogroup C (NmCPS), mice immunized with 50  $\mu$ g of peptide had the best anti-NmCPS response [44]. Thus, the optimal dose depends on the peptide sequence in use and cannot be generalized across peptides. In certain cases, the individual MAP and tripeptide construct dose tested in this study against pneumococcal serotypes may not have been in the optimal range. However, when the combination MAPs or the bipeptide constructs were used, the dosage used appeared to be more optimal for reducing NP carriage. Overall, an obvious trend was observed among all mouse groups immunized with any of the tested peptides—a reduction in NP carriage, regardless of whether it was significant. To look at this in more detail, dose-ranging studies to optimize the effects of these peptides are currently under way.

The use of an appropriate adjuvant also could contribute toward the differences in a peptide response. In the current study, 2 methods were used to elicit an immunogenic response in mice. In the lipidated MAPs, a palmitoyl group was attached to the N-terminal end of the base peptides to enhance immunostimulation. Similarly, for the nonlipidated MAPs, bipeptides, and tripeptide, alum was used as the immunostimulator. Proteosomes have commonly been attached to peptides during peptide synthesis, to increase the immunologic response in animals [25]. The adjuvants palmitic acid, QS21, and alum have been used as immunostimulators in studies and can replace proteosomes as the adjuvant of choice to obtain an immune response [45–47]. Although both palmitic acid and alum were used in the current study to promote an immunologic response, there was no evidence to support one over the other as producing a greater response or better inhibition of NP colonization in the animal model. Although such a hypothesis was not examined during the present study, the problem of adjuvant choice is currently under investigation.

In the present study, PsaA was not used, and, therefore, no direct comparisons between our formulated PsaA peptides and the parent PsaA protein could be made with respect to their effect on NP carriage reduction. However, indirect comparisons can be made through 2 recent studies in which we participated [17, 21] that assessed NP carriage inhibition of *S. pneumoniae* by induced anti-PsaA antibodies. In one study, mice immunized with PsaA and challenged with serotype 6B experienced a 100-fold reduction in carriage, compared with controls [17]. Furthermore, these mice were observed to be colonized on average with ~140 cfu. In the current study, and for serotype 6B, the greatest carriage reduction was a 10-fold, or 91%, decrease and was observed in bipeptide P79-immunized mice. For the other peptide-

immunized mice, reduction in carriage averaged 45%–60%. However, for mice immunized with MAP P46, MAP combinations, and the tripeptide, the numbers of colony-forming units of serotype 6B colonizing the nasopharynx approached those observed in the previous study [17]. In the second study, NP carriage of serotype 2 averaged 30–70 cfu [21]. This was a 10-fold reduction over the control group. In our present study, and for peptide-immunized mice challenged with serotype 2, carriage reduction ranged between 39% and 87%. Furthermore, carriage rates in peptide-immunized mice were generally 10–100-fold higher (284–2516 cfu/mouse) than those observed in the PsaA-immunized mice of the previous study (28–66 cfu/mouse) [21]. In all 3 studies, mice were challenged intranasally with  $10^6$  cfu; however, for one study, the serotype 6B isolate used was not the same one we used in the present study. The ability of PsaA peptide-induced antibodies to inhibit NP pneumococcal carriage was not as robust in our study as was that observed in the 2 previous studies. The differences observed in reduction in carriage between the current and previous studies are not surprising but may have been magnified because of differences in experimental design (e.g., mouse strain, route of immunization, dosage, and adjuvant). In the previous studies, CBA inbred mice were used and were immunized intranasally with 150 or 500 ng of immunogen plus the adjuvant cholera B toxin [17, 21]. On the other hand, ND4 outbred mice were used in our current study and were immunized subcutaneously with 50 or 100  $\mu$ g of immunogen. The adjuvant of choice in this case was either aluminum hydroxide (alum), for nonlipidated mono- and polypeptides, or palmitic acid, for lipidated peptides. Any of these differences, alone or in combination, may have influenced the final outcomes.

The focus of the present study was to examine the effect of various synthesized PsaA peptides on NP pneumococcal carriage in mice. The one common outcome in all cases when mice were immunized with one of the formulated peptides was the observation of reduction in pneumococcal carriage. Even though reduction in carriage was observed in every experiment, it was not always significant ( $P < .05$ ). In some cases, this nonsignificance was most likely due to the sample size ( $n = 8$ ) of the test group (i.e., lack of power). In other cases in which reduction in carriage was low and nonsignificant, increasing the immunization dosage might have enhanced this reduction and led to significant results. Therefore, the most important outcome of this study is not whether reductions in carriage were significant but, rather, that all formulated PsaA peptides were capable of eliciting an immunologic response in mice that, in turn, reduced pneumococcal carriage.

In conclusion, we studied 3 MAPs, either alone in the lipidated or nonlipidated form or in combination with each other, 2 bipeptides, and a tripeptide to determine the inhibitory effect of the induced antibodies on pneumococcal NP carriage in immunized mice. Overall, there was a noticeable inhibitory trend in the effect of immunostimulated antibodies induced to our various pep-

tides on NP carriage. Reduction in carriage was observed for all peptide formulations used to immunize mice. Inhibition of NP carriage was modest in mice immunized with the individual non-lipidated MAPs; however, when lipidated MAPs, MAP combinations, or bipeptides were used, variability was reduced and the ability of the immunostimulated antibodies to inhibit carriage was dramatically enhanced. These observations indicated that the anti-PsaA monoclonal antibody phage display-expressed peptides are immunogenic and reduce NP carriage in mice. These PsaA peptides demonstrate the potential for being important new vaccines against pneumococcal carriage, otitis media, and invasive pneumococcal disease, whether alone or in combination with other vaccine components.

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